Rabex-5 is a lenalidomide target molecule that negatively regulates TLR-induced type 1 IFN production

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Immunomodulatory drugs (IMiDs) are a family of compounds derived from thalidomide. Binding of the IMiD molecule to the Lon protease Cereblon initiates the degradation of substrates via the ubiquitin proteasome pathway. Here, we show that Cereblon forms a complex with Rabex-5, a regulator of immune homeostasis. Treatment with lenalidomide prevented the association of Cereblon with Rabex-5. Conversely, mutation of the IMiD binding site increased Cereblon–Rabex-5 coimmunoprecipitation. The thalidomide binding region of Cereblon therefore regulates the formation of this complex. Knockdown of Rabex-5 in the THP-1 macrophage cell line up-regulated Toll-like receptor (TLR)-induced cytokine and type 1 IFN production via a STAT1/IRF activating pathway. Thus, we identify Rabex-5 as a IMiD target molecule that functions to restrain TLR activated auto-immune promoting pathways. We propose that release of Rabex-5 from complex with Cereblon enables the suppression of immune responses, contributing to the antiinflammatory properties of IMiDs.

IMiD | Cereblon | STAT1 | interferon | TLR

Immunomodulatory drugs (IMiDs) are known for their antiinflammatory properties and are effective in the treatment of certain inflammatory skin disorders (1). The Lon protease Cereblon is the principle receptor for these compounds and following IMiD binding facilitates the recruitment of substrates for ubiquitination by the Cullin4A-associated ubiquitin ligase complex (2, 3). Here, we identify Rabex-5 as an IMiD target molecule. The phenotype of Rabex-5–deficient mice, which spontaneously develop a fatal inflammatory skin disorder (4), taken together with our observations here, strongly suggests the involvement of Rabex-5 in mediating IMiD properties.

The inhibitory effect of IMiDs on Toll-like receptor (TLR)-induced cytokine and type 1 IFN production has been widely noted and is at least one contributing factor to the antiinflammatory properties of IMiDs (5, 6). In particular, IMiDs are noted for their inhibitory effect on LPS-induced TNFα production (7). In contrast to the activity of IMiDs in cancer, which are entirely dependent on Cereblon, these antiinflammtory properties are mediated, at least in part, by Cereblon-independent processes. For example, IMiDs are effective inhibitors of TLR4-induced TNFα, IL12p40, and IFNβ in Cereblon-deficient mice (5). Moreover, thalidomide analogs that lack the Cereblon binding moiety, such as apremilast, retain their inhibitory effect on TLR-induced cytokine production (8).

Nevertheless, several studies support the relevance of Cereblon to the antiinflammatory properties of IMiDs. Ikaros, which is degraded following IMiD treatment, functions as a transcriptional repressor of the regulatory T-cell–promoting cytokine IL2 (9–12). Consequently, treatment with pomalidomide up-regulates IL2 production in a Cereblon-dependent manner (11, 13). Cereblon therefore contributes to the antiinflammatory properties of IMiDs by mediating the degradation of proteins with functions in the immune system. Thus, the immunomodulatory properties of these compounds result from a combination of Cereblon-depedendent and Cereblon-independent factors.

Rabex-5, also referred to as RabGEF1, was first reported as a guanosine nucleotide exchange (GEF) factor with the in vitro capacity to convert Rab5 into its active, GTP-bound form (14). Studies of Rabex-5 function in immunity have shown the Ras family of GTPases to be an important target of Rabex-5 function. Ras is constitutitvely active in Rabex-5–deficient mast cells generated in vitro, resulting in enhanced production of IL6 in response to IgE and antigen stimulation in vitro (4). Therefore, Rabex-5 maintains immune homeostasis by preventing excessive Ras-induced immune responses. Here, we find that Rabex-5/Ras signaling regulates TLR-induced STAT1/IRF activating pathways. We propose that the IMiD-induced release of Rabex-5 from complex with Cereblon contributes to the antiinflammatory properties of these compounds by enabling the suppression of immune responses.

Results

Cereblon Specifically Interacts with Rabex-5. To identify potential effectors of IMiD properties, we prepared expression vectors for established regulators of immunity and performed interaction studies with recombinant Cereblon in HEK293T cells. As part of this analysis, we included proteins with homology to the inflammatory regulator A20 (15). Among these proteins, Cereblon was found to specifically coimmunoprecipitate with Rabex-5 (Fig. S1*A*). To investigate the physiological relevance of Cereblon–Rabex-5 binding, we performed coimmunoprecipitation (co-IP) studies using endogenous protein in the murine RAW264.7 cell line. Cereblon coimmunoprecipitated with Rabex-5 at above background levels (Fig. S1*B*). Rabex-5 is therefore capable of associating with Cereblon in the absence of recombinant

Significance

The identification of the Lon protease Cereblon as the thalidomide receptor has led to significant progress in our understanding of immunomodulatory drugs (IMiDs). IMiD binding alters the surface of Cereblon to facilitate the recruitment of proteins including Ikaros and CK1α. The recruitment of these proteins results in their ubiquitin-mediated proteosomal degradation. How Cereblon mediates the efficacy of these compounds in the treatment of inflammatory skin conditions remains unclear. Significantly, this study has identified Rabex-5 as a Cereblon-interacting protein that is affected by IMiD binding. Furthermore, knockdown studies in a human macrophage cell line reveals a function of Rabex-5 in regulating Toll-like receptor–induced type 1 IFN production. These findings may help explain the antiinflammatory properties of IMiDs.

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overexpression. Given the rapid degradation of $CUL4A^{CRBN}$ substrates following IMiD treatment, Cereblon–substrate protein interactions are likely to be difficult to detect in the absence of proteosomal inhibition. Addition of the proteasome inhibitor MG132 markedly up-regulated Cereblon–Rabex-5 binding (Fig. S2). Mutation analysis identified amino acids 201–250 as critical for Rabex-5 binding (Fig. S3). This region is within the DDB1 binding portion and serves to connect Cereblon to the wider $CUL4A^{CRBN}$ complex (13). Collectively, these data identify Rabex-5 as a specific (among A20 homology proteins) $CUL4A^{CRBN}$-interacting protein. We note that protein levels of recombinant Rabex-5 were unaffected by Cereblon overexpression or the addition of MG132 (Figs. S1 and S2).

Lenalidomide Prevents the Association of Cereblon with Rabex-5. Next, we investigated how IMiD binding might affect the Cereblon–Rabex-5 interaction. The addition of high concentrations of lenalidomide (200 μM) completely eliminated Cereblon–Rabex-5 binding (Fig. 1*A*). The decrease in Cereblon–Rabex-5 binding correlated with increasing doses of lenalidomide in a nonlinear manner (Fig. 1*B*). These data indicate that $CUL4A^{CRBN}$ forms a complex with Rabex-5 that is disrupted by lenalidomide. Mutation of tyrosine and tryptophan residues at positions 384 and 386, respectively, to alanine (YW/AA) is reported to reduce IMiD binding and rescue thalidomide teratogenicity (16). The interaction between $CRBN^{YWAA}$ and Rabex-5 was resistant to the effects of IMiDs. Furthermore, we observed a threefold increase in basal levels of Cereblon–Rabex-5 binding (Fig. 2*A*). These data show that disruption of Cereblon–Rabex-5 binding is a direct result of lenalidomide binding to Cereblon. We note that lenalidomide was substantially more effective at reducing Cereblon–Rabex-5 binding than either thalidomide or pomalidomide (Fig. 2*A*). Moreover, disruption of the complex had no effect on protein levels of recombinant Rabex-5 (Figs. 1*A* and 2*A*).

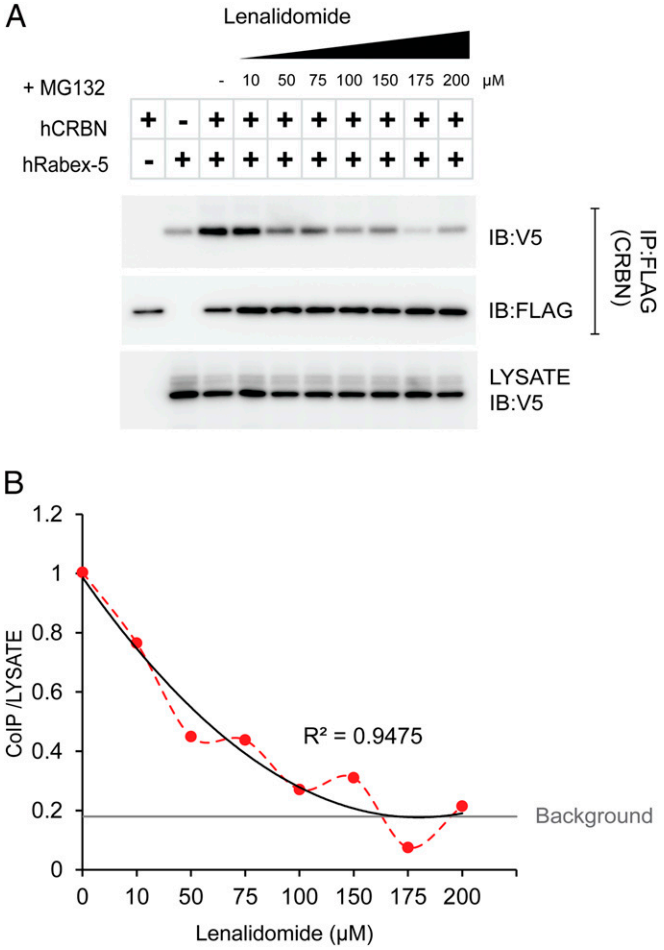

Fig. 1. Lenalidomide prevents the association of Cereblon and Rabex-5. (*A*) HEK293 cells were transfected with full-length human recombinant FLAG-tagged Cereblon and V5-tagged Rabex-5 expression vectors. Lenalidomide was added 2 h before the addition of MG132. At 4 h after the addition of MG132 (6 h after IMiD treatment), cells were lysed. Immunoprecipitated and lysate (input) samples are as indicated. (*B*) Band intensity in *A* was measured and plotted as the ratio Co-IP/lysate. Red dotted line shows actual data points. Trend line is shown as solid black line. Gray line indicates background levels of Rabex-5 co-IP. R2 value indicates quality of the trend line. For example, a value of 1.0 indicates perfect correlation between *x* and *y* axes. Data are representative of at least three independent experiments.

The IMiD-induced degradation of substrates does not occur in murine systems (13, 17). Therefore, we next compared the effect of IMiD treatment on the Cereblon–Rabex-5 interaction using human and murine recombinant protein. The increase in Cereblon–Rabex-5 binding caused by the YW/AA mutation was observed in both systems, whereas only human Cereblon was sensitive to the effects of IMiDs (Fig. 2 *A* and *B*). Thus, the protein interaction, in addition to the regulatory effect of Y384 and W386 on Rabex-5 binding, is conserved between humans and mice. However, consistent with other reports, only human Cereblon is responsive to IMiD binding (13, 17).

Rabex-5 Negatively Regulates TLR-Induced Type 1 IFN Production. To understand how Rabex-5 might contribute to the antiinflammatory properties of IMiDs, we next investigated the function of Rabex-5 in cytokine production induced via TLR4. Rabex-5–deficient macrophages were generated by the stable transfection of THP-1 cells with nontargeting (NT) or Rabex-5 targeting shRNA. Effective knockdown was assessed by RT-PCR and Western blotting (Fig. 3 *A* and *B*). To assess the effect of Rabex-5 deficiency on signaling pathways, we used commercially available THP-1 cells modified to secrete alkaline phosphatase and luciferase in response to NF-κB and IFN-stimulated response element (ISRE) (IRF and STAT1/STAT2 binding promoter sequence) activation, respectively (Invivogen).

Rabex-5 deficiency strongly up-regulated basal and LPS-stimulated levels of ISRE induction. A modest increase in basal NF-κB activation was also observed. The production of inflammatory cytokine IL6, but not TNFα, was robustly augmented by Rabex-5 knockdown. IP10, an IRF-regulated chemokine, was also up-regulated (Fig. 3*A*). IFN-β and IP10 mRNA was markedly increased in Rabex-5–deficient cells in response to stimulation with LPS, as assessed by RT-PCR (Fig. 3*B*). ISRE activation was enhanced by Rabex-5 deficiency following stimulation with agonists for TLR7 and in response to stimulation with IFNα (Fig. 3*C*). THP-1 cells failed to mount a robust response to agonists for TLR3 and TLR9; however, a small increase in ISRE induction was observed downstream of these receptors. Furthermore, protein levels of IFNα produced in response to ligands for TLR4 and TLR7, and IFNβ in response to TLR7, was markedly increased as assessed by ELISA (Fig. 3*D*). We note that the increase in basal levels of NF-κB activation was limited to THP-1 cells in their macrophage/phorbol 12-myristate 13-acetate (PMA) differentiated state and is therefore likely a result of the differentiation process (Fig. 3 *A* and *C*). Western blot analysis revealed no effect on TLR4-induced TBK1, IKK-α/β, or p65 phosphorylation; however, STAT1 was constitutively phosphorylated in Rabex-5–deficient cells (Fig. S4). Furthermore, the basal and LPS-induced increase in ISRE induction was completely absent in $MYD88^{-/-}$ cells (Fig. S4). Collectively, these data suggest that Rabex-5 negatively regulates type 1 IFN production via a MYD88-dependent STAT1 activating pathway.

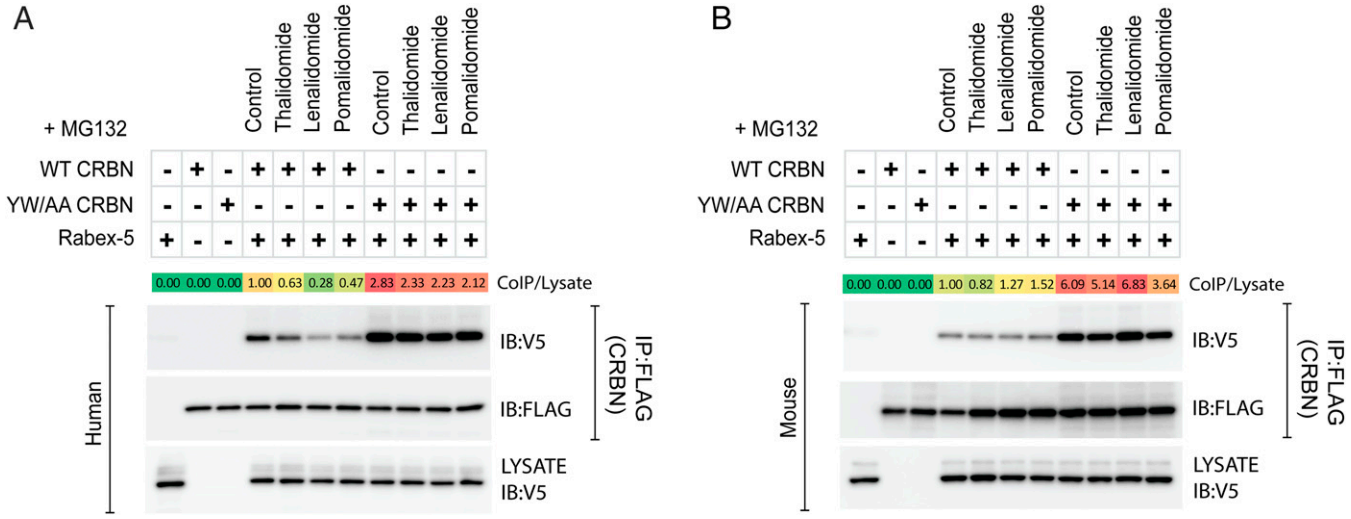

Fig. 2. Comparative analysis of Cereblon–Rabex-5 interaction in human and murine systems. (*A*) Human recombinant Flag-tagged WT Cereblon, $CRBN^{YWAA}$, and V5-tagged Rabex-5, were transfected into HEK293T cells as indicated. IMiD treatments were performed as in Fig. 1. IP and lysate (input) samples are as indicated. Band intensity of Rabex-5 immunoblot was measured and expressed as a ratio of Co-IP/lysate. Data are shown as color coded table (red, high CoIP; green, low CoIP). Cells were treated with 200 μM thalidomide/lenalidomide or 100 μM pomalidomide as indicated. (*B*) Same experiment as in *A* using murine recombinant Cereblon and Rabex-5. Data are representative of at least three independent experiments.

Discussion

The antiinflammatory properties of thalidomide derivatives have long been appreciated (6). For example, in addition to the in vitro inhibitory effect of these compounds on TLR-induced cytokine and type 1 IFN production (5, 7), clinical studies have shown IMiDs to be effective in the treatment of immunological disorders, including cutaneous lupus erythematous and multiple sclerosis (18, 19). However, although the therapeutic potential of IMiDs is widely recognized, concerns over teratogenicity have restricted their wider use in the treatment of sublethal diseases, including inflammatory and autoimmune disorders. The mechanism underlying these properties, and in particular the contribution of Cereblon, is therefore of substantial interest.

Our previous investigations have revealed that at least some of these properties, including the suppression of TNFα, are in fact mediated separately from Cereblon (5). Nevertheless, the antiinflammatory properties of IMiDs are unlikely to be fully explained by Cereblon-independent processes, as the inhibitory effect on TNFα is limited to 30–40% as assessed following TLR4 stimulation (5, 7). Moreover, the Cereblon-mediated degradation of Ikaros and other substrates are certain to contribute to the overall effects of these compounds in human systems. Here, we have identified Rabex-5 as a Cereblon-interacting protein and IMiD target molecule. Our observations on the disruption of Cereblon–Rabex-5 binding differ substantially from previously identified interactions with Ikaros, CK1α, GS, or MEIS2 (10, 17, 20–22). In particular, protein levels of Rabex-5 were not affected by loss of Cereblon binding. The biological significance of our observation therefore remains unclear. Moreover, in practice, showing that IMiDs can affect Rabex-5 regulated pathways is complicated by the various Cereblon-independent effects that can be observed (5). Thus, at present, we can only speculate that the regulatory effect of Rabex-5 on TLR-induced type 1 IFN production is relevant to the properties of IMiDs. Nevertheless, based on our observations, we propose that IMiDs may promote immune homeostasis by enabling the Rabex-5–mediated suppression of immune responses.

This is by no means the only mechanism by which IMiDs might interfere with autoimmune-promoting pathways and in particular the suppression of type 1 IFN production. For example, if we consider the efficacy of IMiDs in the treatment of cutaneous lupus erythematous, there are likely to be several contributing factors: the Cereblon-independent suppression of IFNβ (5); the Cereblon-mediated degradation of Ikaros (12); and potentially, the mechanism described here. Future work is required to understand the relative importance of these processes in mediating the antiinflammatory properties of IMiDs. In particular, the contribution of Rabex-5 to mediating these effects will depend on the extent to which Cereblon and Rabex-5 are able to associate with one another in a given cell type. For instance, although we were able to observe clear binding of endogenous Cereblon and Rabex-5 in RAW264.7 cells, obtaining data in primary cells proved difficult. Therefore, it seems likely that the interaction between Cereblon and Rabex-5 may be restricted to certain cell types or conditions, or alternatively, may be limited to a particular subcellular location. Given what is known about the function of Rabex-5, interaction at the endosomal membrane seems likely. We suggest that further analysis of the interaction may be achieved using cell fractionation and/or fluorescent microscopy.

The precise functional relationship between Cereblon and Rabex-5 has been difficult to determine. Given the literature (20, 21, 23, 24), we considered whether Rabex-5 is a substrate of $CUL4A^{CRBN}$. However, protein levels of recombinant Rabex-5 were unaffected by IMiD treatment. Furthermore, although Y384 and W386 were able to regulate Rabex-5 binding, the Cereblon–Rabex-5 interaction occurred independently of the IMiD binding region of Cereblon. Therefore, protein levels of Rabex-5 do not appear to be regulated by Cereblon.

Because Rabex-5 is not a substrate of $CUL4A^{CRBN}$, we speculate that the interaction may have some functional significance. To understand this relationship, we must explain two observations made in this study: (*i*) IMiD binding prevents the Cereblon–Rabex-5 interaction, and (*ii*) $CRBN^{YWAA}$ is more readily able to bind Rabex-5 compared with WT CRBN. We suggest that these two observations might be explained if the binding of substrates to Cereblon prevents the formation of a complex with Rabex-5. Thus, according to this hypothesis, the first observation is explained by the IMiD-induced recruitment of substrates IKZF1, IKZF3, and CK1α (17, 20, 23), perhaps resulting in a conformational change that renders Cereblon incapable of binding Rabex-5. This system is present only in human systems. Similarly, the second observation can be explained by the reduced binding of $CRBN^{YWAA}$ to

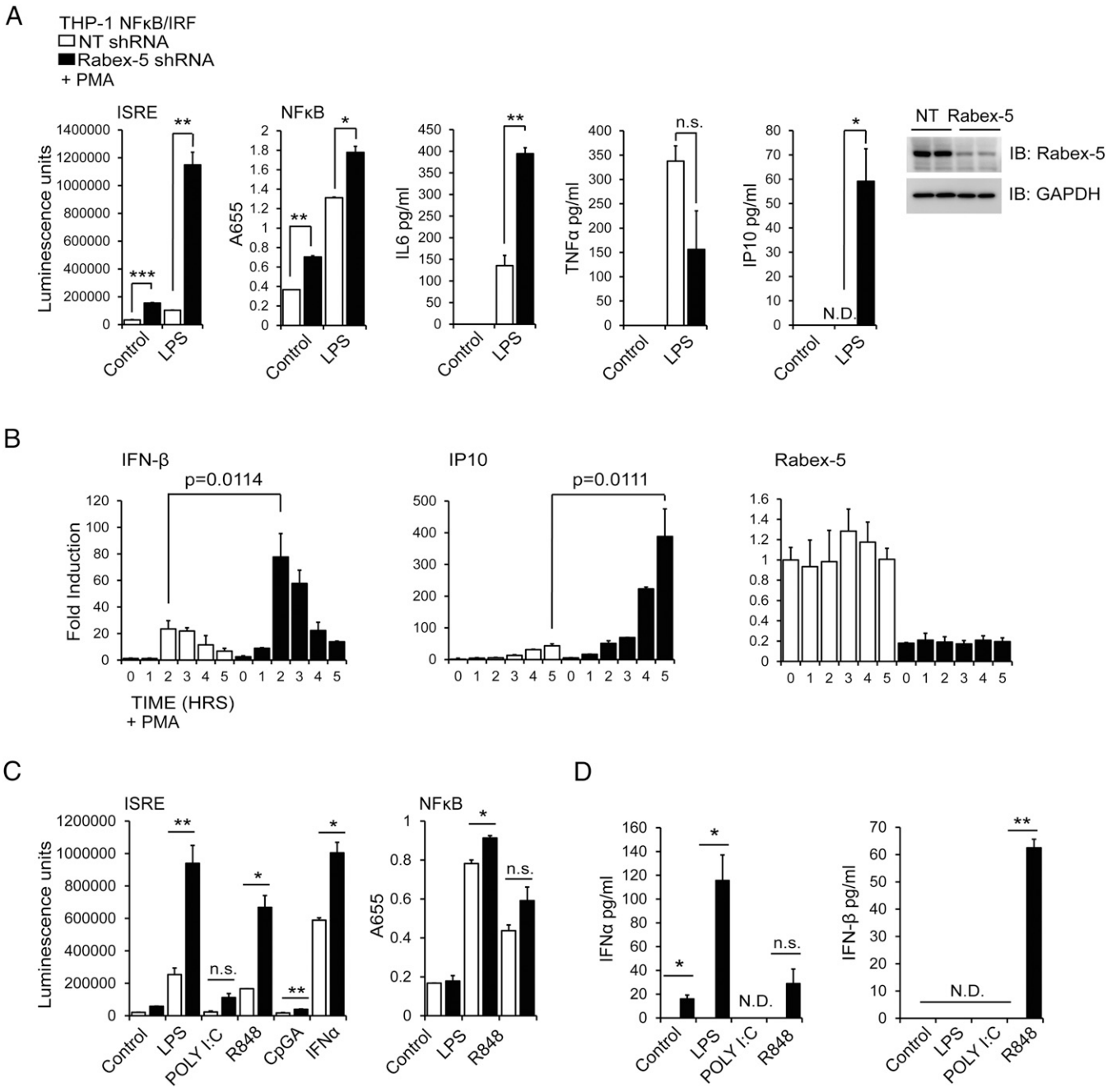

Fig. 3. Rabex-5 negatively regulates TLR-induced type 1 IFN production. (*A*) THP-1 cells were stably transfected with NT or Rabex-5 targeting shRNA, differentiated with PMA and stimulated with LPS overnight (16 h). Cytokine levels were measured in culture supernatant by ELISA. Luciferase expression (ISRE) was measured from culture supernatant and is expressed in luminescence units. Alkaline phosphatase (NF-κB) was measured at absorbance filter A655. Knockdown of Rabex-5 was confirm by Western blot analysis (*Right*). (*B*) Time course analysis of LPS stimulated, PMA differentiated THP-1 cells stably transfected with NT or anti–Rabex-5 shRNA. Cytokine production was measured by quantitative real-time PCR analysis and expressed as fold induction over unstimulated samples. Rabex-5 mRNA was measured in the same samples and is shown (*Right*). (*C*) Rabex-5 negatively regulates TLR- and IFNα-induced ISRE induction. Stimulations performed O/N as before. (*D*) Protein levels of IFNα and IFNβ measured by ELISA. Stimulations were performed overnight. Cells were not differentiated with PMA (*C* and *D*). Error bars show the SD of the mean of two replicate wells (two separate aliquots of cells treated with the same stimulations; $n = 2$). Data are representative of at least three independent experiments. *P* values were calculated using Student's *t* test (***$P < 0.005$, **$P < 0.05$, *$P < 0.1$, n.s. $P > 0.1$). Where cytokine levels could not detected by ELISA, this is indicated as not detected (ND).

glutamate synthetase (GS). GS is the first $CUL4A^{CRBN}$ substrate to be identified that does not require IMiD binding to associate with Cereblon. GS is (to an extent) always bound to Cereblon and this binding is conserved between humans and mice. Moreover, GS is unable to bind $CRBN^{YWAA}$ (21). Therefore, the absence of GS binding in the $CRBN^{YWAA}$ mutant may explain the increase in basal levels of Rabex-5 binding. In short, we propose that substrate binding to Cereblon prevents the formation of a protein complex with Rabex-5.

Mechanistically, Rabex-5 is reported to regulate immune responses via the negative regulation of RasGTPase activated pathways (4, 25, 26). Previous studies have focused on the function

of Rabex-5 in mast cells, suggesting that the phenotype of Rabex-5–deficient mice might reflect, at least in part, defective mast cell function (4, 25, 26). Here, we have reported that Rabex-5 is a critical negative regulator of type 1 IFN production. Based on the strength of our observations in vitro, we suggest that excessive type 1 IFN production may be the driving factor of the spontaneous inflammatory disorder exhibited by Rabex-5–deficient mice. Indeed, increased histamine and IgE serum levels, reported in Rabex-5–deficient mice, are also characteristics of type 1 IFN-mediated disorders such as systemic lupus erythematous (4, 27). This conclusion is further supported by the numerous studies that have linked STAT1, which we have observed to be overactive in Rabex-5–deficient cells, to this disorder (28). Future studies may address the importance of this observation by investigating whether STAT1 deficiency rescues the phenotype of Rabex-5–deficient mice.

In this study, we considered whether the endosomal function of Rabex-5 was an adequate explanation for these observations. Following receptor ligation TLR4 undergoes an endocytosis and trafficking process and is ultimately destined for degradation in the lysosome (29). Whereas the relevance of these processes to STAT1 is unclear, the activation of IRF3 and the production of type 1 IFN, induced via TLR4, is known to require endosomal maturation (30). One possibility is that endocytosis/trafficking could be delayed in the absence of Rabex-5, resulting in prolonged signal transduction. Rabex-5 is one of several proteins capable of driving endosomal maturation via the Rab5 GTPase (31) and it may be that one of these proteins is able to partially compensate for the loss of Rabex-5, perhaps with reduced efficiency, resulting in a longer TLR4 transit period and enhanced STAT1 (and possibly IRF3) activation at the endolysosome. Such processes have previously been described to affect type 1 IFN production. For example, prolonged retention of CpGA in the endolysosome is known to increase IFN-α production induced via TLR9 (32).

Our observations using fluorescent microscopy support the relevance of these processes in mediating the effects of Rabex-5 deficiency. For example, Rabex-5 could be observed at the outer membrane of LAMP1-positive (late endosomal) vesicles in LPS-stimulated macrophages. The nuclear translocation of Rabex-5 was also evident. Thus, Rabex-5 is present in TLR4 containing late endosomes and eventually translocates to the nucleus. Although the function of the lysosomal compartment was not impaired by Rabex-5 deficiency, the increase in ISRE induction was highly sensitive to addition of the endosomal inhibitor chloroquine (Figs. S5 and S6). Therefore, endosomal processes are evidently relevant to the Rabex-5–mediated control of type 1 IFN production.

Whereas the effect on type 1 IFN production can be readily explained by the putative endosomal function of Rabex-5, it is not immediately clear why these processes would have opposing effects on TLR4-induced IL6 and TNFα production. On this point, our results differ from published observations. Specifically, physiological mast cells, derived in vitro from Rabex-5–deficient mice, are reported to produce increased levels of both IL6 and TNFα in response to IgE and antigen challenge (4). Therefore, we suggest that reduced levels of TNFα production seen in our experiments (Fig. 3*A*) may reflect a peculiarity of the THP-1 cell line or a difference in Rabex-5 function between macrophage and mast cell lineages. Alternatively, increased STAT1 signaling may negatively impact other signaling pathways activated downstream of TLR4, perhaps by sequestering resources and/or signaling components with functions in multiple pathways. Nevertheless, the function of Rabex-5 in vivo is overwhelmingly antiinflammatory, and therefore, our observation of reduced TNFα production appears to lack physiological relevance. Experiments in primary cells will be required to address this point.

In addition to its endosomal function, another possibility is that Rabex-5 regulates immune responses via its negative regulatory function on the Ras family of GTPases. So far only one study has addressed the role of Ras in TLR signaling. Specifically, N-Ras was found to promote IL6 production via MAPK pathways (33). Consistent with this study, we found that chemical inhibition of Ras using the farneslytransferase inhibitor salirasib suppressed TLR-induced ISRE induction (Fig. S7). Ras is therefore a positive regulator of TLR responses in vitro. Furthermore, the in vivo relevance of these observations is supported by the involvement of Ras in a rare type of autoimmunity (34, 35). These two mechanisms: the promotion of endosomal maturation and the suppression of Ras signaling, are not mutually exclusive. For example, it may be that prolonged endosomal maturation is the cause of increased Ras activation, which in turn promotes STAT1 signaling/IL6 production. Whatever the precise mechanism, we expect that a greater understanding of Rabex-5/Ras/STAT1 signaling is likely to shed light on mechanisms functioning to prevent excessive type 1 IFN production and the development of autoimmune disease.

The contribution of these processes to mediating the effects of IMiDs remains unclear; however, we have shown that in principle, there is potential for an IMiD-sensitive relationship between Cereblon and Rabex-5, which in turn has the potential to contribute to the antiinflammatory properties of IMiDs. Further study is required to understand the functional significance of this interaction in addition to the importance of Rabex-5 in mediating the antiinflammatory properties of thalidomide derivatives.

Materials and Methods

Chemicals. All chemicals were reconstituted in DMSO. Thalidomide (Sigma, T144) lenalidomide (LKT Laboratories, L1852) were reconstituted to a stock concentration of 50 mg/mL pomalidomide (Sigma, P0018) and salirasib (Cayman Chemical Company, 10010501) were reconstituted to a concentration of 10 mg/mL and 20 mg/mL, respectively. MG132 (Calbiochem, 474790) was reconstituted to a concentration of 10 mM. For stimulations, DMSO did not exceed a final concentration of 0.5%. Final concentrations are indicated in the main text and figures. Chloroquine diphosphate was purchased from Nacalai Tesque (08660-04) and reconstituted in water to a concentration of 50 mg/mL.

Stimulations/TLR Agonists. *Salmonella*-derived LPS was purchased from Sigma (L6261). Other TLR ligands were purchased from Invivogen (PAM3CSK4; tlrl-pms, R848; tlrl-848–5, human CpGA; ODN2216, high molecular weight Poly I:C; tlrl-pic). Recombinant human IFNα (Alpha 2a) was purchased from R&D Systems (11100-1). Where indicated, THP-1 cells were differentiated into a macrophage state by the addition of PMA (Nacalai Tesque, 27547–14). Twenty-four hours after PMA treatment (50 ng/mL), fresh medium was added. Stimulations were performed 48 h after PMA treatment. Unless otherwise indicated, cells were stimulated overnight with TLR ligands (16–24 h). We used the following final concentrations for stimulation: LPS, 1 μg/mL; PAM3CSK4, 300 ng/mL; R848, 10 μg/mL; CpGA, 5 μM; and Poly I:C, 10 mg/mL. Recombinant IFNα was used at a concentration of 2 μg/mL.

ELISA. Cytokine production was measured from culture supernatant as per the manufacturer's instructions: Biolegend human IL6 (430504) TNFα (430204), and IP10 (439904). Human IFNα and IFNβ was measured using the Verikine ELISA kit available from R&D Systems (PBL Assay Science, 41100, 41410-1). For details of NF-κB/ISRE measurements, see *SI Materials and Methods*.

Quantitative Real-Time PCR Analysis. RNA was prepared using the RNeasy Mini kit (Qiagen). cDNA was prepared using the QuantiTect Reverse Transcription kit (Qiagen). Quantititative real-time PCR was performed on an ABI PRISM 7900HT (Applied Biosystems). Fast start universal SYBR Green master mix (Roche) was used together with the following primers: hRabex-5, forward 5′-GGAAATTCAGGAAGCAAAAG-3′ and reverse 5′-AACTCCTTAGACACTCTATCC-3′; hIFNβ, forward 5′-GGCAATTGAATGGGAGGCT-3′ and reverse 5′-GGCGTCCTCCTTCTGGAACT-3′; hIP10, forward 5′-TGACTCTAAGTGGCATTCAAGGAG-3′ and reverse 5′-TTTTTCTAAAGACCTTGGATTAAC; h18S, forward 5′-GGGAGGTAGTGACGAAAAAT-3′ and reverse 5′-ACCAACAAAATAGAACCGCG-3′. cDNA was normalized to 18S rRNA and expressed as fold induction over unstimulated samples.

Lentiviral Infection. Nontargeting control shRNA and anti–Rabex-5 shRNA constructs were purchased from Sigma-Aldrich (MISSION shRNA anti-Rabgef1, TCRN000004733; control shRNA, SHC202). Lentivirus was prepared using MISSION lentiviral packaging mix (Sigma, SHP001). Briefly, 2.5 μg shRNA vector was mixed with 26 μL packaging mix and transfected into HEK293 cells (10-cm dish) using lipofectamine 2000 (15 μL). After 48 h, virus-containing supernatant was collected and passed through a 45-μm filter (Millex). THP-1 cells were resuspended in virus-containing medium and subjected to spin transfection at 900 × *g* for 90 min at room temperature. Cells were rested for 4 h at 37 °C and then replated in fresh medium. After 24 h, puromycin (10 μg/mL) was added to the medium. Cells were expanded under selection for at least 1–2 wk before stimulation.

Plasmids. Murine Cereblon cDNA was amplified from primary peritoneal macrophages (C57BL/6) and cloned into the p3xFLAG-CMV-7.1 vector (Sigma) using directional cloning with BamHI and XbaI extended primers. The YWAA mutation was introduced using the KOD-Plus mutagenesis kit (Toyobo). Human and murine Rabex-5 were cloned using the pcDNA3.1-V5-His TOPO directional cloning system. Human Cereblon and YWAA mutant were a generous gift from Hiroshi Handa and Takumi Ito, Tokyo Insitute of Technology, Tokyo. Details have been described previously (16). Plasmids were checked by sequencing. Detailed methods on immunoprecipitation experiments can be found in *SI Materials and Methods*.

Statistics. *P* values were calculated using the Student *t* test.

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